Species Discrimination of Postlarvae and Early Juvenile Brown Shrimp (*Farfantepenaeus aztecus*) and Pink Shrimp (*F. duorarum*) (Decapoda: Penaeidae): Coupling Molecular Genetics and Comparative Morphology to Identify Early Life Stages

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SPECIES DISCRIMINATION OF POSTLARVAE AND EARLY JUVENILE BROWN SHRIMP (*FARFANTEPENAEUS AZTECUS*) AND PINK SHRIMP (*F. DUORARUM*) (DECAPODA: PENAEIDAE): COUPLING MOLECULAR GENETICS AND COMPARATIVE MORPHOLOGY TO IDENTIFY EARLY LIFE STAGES

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ABSTRACT

We collected postlarvae (PL) and early juveniles of *Farfantepenaeus aztecus* and *F. duorarum* < 7.0 mm CL from the Gulf of Mexico and verified their species identity using a multiplex Polymerase Chain Reaction (PCR) assay, which targeted the 16S rRNA mitochondrial gene. We examined young with ≥ 5 dorsal teeth (DT) for differences in morphology and used a General Discriminant Analysis approach and 'best' subsets model-building technique to help identify the 'best' characters to discriminate taxa and predict species membership. *Farfantepenaeus duorarum* with ≥ 5 DT and *F. aztecus* with ≥ 7 DT have spinules on the epigastric and first DT, a character not previously reported for these two species. Differences in antennal scale shape and sixth pleomere length discriminate > 95% of *Farfantepenaeus* sp. with < 7 – 8 + 2 rostral teeth. *Farfantepenaeus duorarum* has an antennal scale with an acutely rounded margin about twice the height of the adjacent lateral spine, and has a sixth pleomere length measurement < 2.5 mm. In *F. aztecus*, the antennal scale has a more broadly rounded margin with a lateral spine that approaches or exceeds the tip of the scale, and has a sixth pleomere length measurement > 2.5 mm. Species discrimination of *Farfantepenaeus* sp. with ≥ 7 – 8 + 2 rostral teeth requires body measurements. Classification models accurately discriminate > 90% of *Farfantepenaeus* sp. from the western Gulf and increase the reliability of discrimination by > 20% over characters that have been used for species discrimination, some of which are unreliable. The unsatisfactory performance of the models in discriminating *Farfantepenaeus* sp. from the eastern Gulf is consistent with the possibility of different ecological populations in the eastern and western Gulf that may warrant further study. Integration of molecular taxonomy and comparative morphology, as we did here, can provide insight into the patterns of diversity and ecological and evolutionary principles that encompass fisheries management.

KEY WORDS: *Farfantepenaeus*, General Discriminant Analysis, Gulf of Mexico, multiplex PCR assay, spinules

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INTRODUCTION

The domestic shrimp fishery, concentrated primarily in the Gulf of Mexico (Gulf), is the second most valuable fishery in the U.S. (Anonymous, 2004). Historically, the Gulf region has provided about 80% by weight and 85% by value of all shrimp landed in the U.S. annually (Anonymous, 2004). The Gulf shrimp fishery consists primarily of three commercial species, brown shrimp, *Farfantepenaeus aztecus* (Ives, 1891), also known as *Penaeus aztecus*; pink shrimp, *F. duorarum* (Burkenroad, 1939), also known as *P. duorarum*; and, white shrimp, *Litopenaeus setiferus* (Linnaeus, 1767), also known as *P. setiferus*. Here, we follow the taxonomy of Pérez-Farfante and Kensley (1997) and McLaughlin et al. (2005) and use *Farfantepenaeus* and *Litopenaeus* to designate genera despite ongoing debate about the generic or sub-generic classification of the group formerly known as *Penaeus* (Flegel, 2007). *Farfantepenaeus aztecus* occur primarily west of the Mississippi River through Tamaulipas, Mexico, whereas *F. duorarum* occur primarily along the Gulf coasts of Florida and Mexico (Pérez-Farfante, 1970) with a smaller population off south Texas. *Litopenaeus setiferus* occur primarily between the northern panhandle of Florida and coastal bend of Texas (Nance et al., 1989).

All three commercially important penaeids spawn demersal eggs in coastal waters usually < 50 m deep. After hatching, larvae become planktonic and transit a series of developmental stages, i.e., nauplius, protozoea, mysis, as tidal and wind-driven currents carry the larvae shoreward. Young eventually enter the estuary as post-larvae (PL's) at about 7-9 mm total length [TL] (Copeland and Truitt, 1966; Baxter and Renfro, 1967), and move into coastal marshes to settle in preferred habitat where they feed and grow rapidly (Dall et al., 1990a). Young move into the open bay as sub-adults and later migrate offshore to join the adult population.

Coastal marshes are an important link to fishery production for estuarine-dependent species and provide food and shelter for early life stages that can lead to increased rates of survival, growth and productivity (Zimmerman et al., 2000; Minello et al., 2008). Large annual fluctuations in size of penaeid stocks suggest a complex relationship between spawning stock size and recruitment (Garcia, 1983; Ye, 2000). An index of relative abundance or catch per unit area derived from the number

Table 1. Characters that have been used to discriminate *Farfantepenaeus duorarum* and *F. aztecus*. Divide total length (TL) by five to approximate carapace length (CL). [1] Males differ in shape of sternite XIV posteromesial ridge. [2] Refer to Pérez-Farfante (1970) for specific details of shape differences between taxa.

mm TL	Character	*F. aztecus*	*F. duorarum*	Literature
< 12	Antennal spine nearly reaches or exceeds tip of antennal scale	Yes	No	Williams (1959); Dobkin (1961)
< 12	Rounded shape of antennal scale margin	Broad	Acute	Williams (1959); Dobkin (1961)
> 17	Length to depth ratio of rostrum	Longer/Narrower	Shorter/Wider	Williams (1953)
> 18	Shape of posterior margin of sternite XIII	Generally straight	Slightly convex	Pérez-Farfante (1970)
> 20	Elevation of lateral margin of sternite XIV (females) [1]	Incomplete	Complete	Pérez-Farfante (1970)
> 20	Distribution of chromatophores on uropods	Concentrated distally	Scattered uniformly	Williams (1953)
> 35	Rostrum extends beyond distal margin of third antennular segment	No	Yes	Pérez-Farfante (1970)
> 35	Shape of median and lateral lobes of petasma and ventral costa (males) [2]	Indistinct	Distinct	Pérez-Farfante (1970)
> 35	Width of dorsolateral sulcus of carapace and sixth pleomere; height of keel	Wide; High	Narrow; Low	Pérez-Farfante (1970)

of PL and early juvenile shrimp in nursery areas provides an estimate of year-class strength (Baxter and Renfro, 1967). Dependable estimates of year-class strength and a better understanding of the ecological parameters, habitat preferences, and distribution patterns of shrimp, however, require reliable species discrimination of young while in estuarine nurseries (Pérez-Farfante, 1970; Rothlisberg et al., 1983). Incorporation of species-specific biological and environmental parameters into stock assessment models provides fishery managers with an estimate of annual stock size (Waples et al., 2008; Reiss et al., 2009).

Characters that discriminate early life stages (generally nauplii through first or second PL), and larger juveniles and sub-adults have been described for the commercially important members of the Family Penaeidae, but reliable species discrimination of later PL and early juveniles remain problematic. While larvae of *F. aztecus* and *F. duorarum* have been laboratory-reared and their early development described from known parentage (Dobkin, 1961; Cook and Murphy, 1971; Kitani, 1985), characters that discriminate PL and early juvenile *F. aztecus* and *F. duorarum* during their period of estuarine residency are largely arbitrary and subjective (Table 1). Differences in seasonal occurrence of *F. aztecus* and *F. duorarum* can assist with species identification when recruitment periods are distinct, as in the Atlantic Ocean (Williams, 1959), but spawning seasons overlap in the Gulf and young often co-occur in estuarine nurseries (Cook and Murphy, 1971).

Recent advances in molecular techniques have improved our ability to identify and discriminate morphologically similar aquatic organisms (Baldwin et al., 1998; Maggioni et al., 2001; Lavery et al., 2004) and assess differences, and permits verification of species identity regardless of life stage. Molecular (Palumbi and Benzie, 1991; Powell et al., 1997; Maggioni et al., 2001) and morphometric approaches (Chuensri, 1968; Heales et al., 1985; May-Kú et al., 2006) have been applied independently to problems in penaeid taxonomy. Only Pendrey et al. (1999) combined genetics with comparative morphology to confirm identity and define species-specific characteristics to discriminate shrimp. Our objective was to molecularly verify the species identity of PL and early juvenile *F. aztecus* and *F. duorarum* and examine these stages for differences in morphology. Our goal was to identify a reliable suite of morphological characters that resolve objectively the species identity of PL and early juvenile *F. duorarum* and *F. aztecus*.

Materials and Methods

We collected PL and early juvenile shrimp by hand-net and benthic sled in the western Gulf from late-February through mid-December and preserved specimens in 70% non-denatured ETOH. Collections were concentrated in the Galveston Bay area, but extended from the mouth of Calcasieu Lake, Louisiana, southwestward to Port Isabel, Texas (Fig. 1). We also examined shrimp collected in the eastern Gulf near Panama City and Tampa, Florida, and in Florida Bay (Fig. 1). We define the eastern and western Gulf as the area bisected by the Mississippi River Delta.

We made a suite of measurements (Appendix Table 1) on the left side of the body (unless appendages were damaged or missing) to the nearest 0.01 millimeter (mm) under a stereozoom microscope with Image-Pro Express 6.0 measurement software. We removed muscle from the abdomen and sent this tissue to the Molecular Ecology and Fisheries Genetics (MEFGEN) laboratory at Texas A&M University, Galveston, for verification of species identity using a multiplex PCR assay, which targeted the 16S rRNA mitochondrial gene as described in Alvarado Bremer et al. (2010). We counted the number of teeth along the rostrum dorsally (DT) and ventrally (VT) to evaluate potential differences between species in the timing of rostral tooth development, and measured the distance between teeth, i.e., inter-tooth distances. Penaeids add teeth sequentially in a posterior to anterior direction as the rostrum elongates. We counted only teeth with the spinous tip 'free' from the shaft of the rostrum, i.e., not nubs, and do not include the epigastric tooth in total counts. We included only PL's with $\geq$ 5 DT in statistical analyses, but included several specimens with four DT in discussions for comparison purposes.

We used Cluster Analysis (StatSoft, 2004) to organize observed data into meaningful structures and group shrimp from the western Gulf into data sets based on morphological similarity and carapace length (CL). We then used a General Discriminant Analysis (GDA) approach and 'best' subsets model-building technique (StatSoft, 2004) on each data set to help

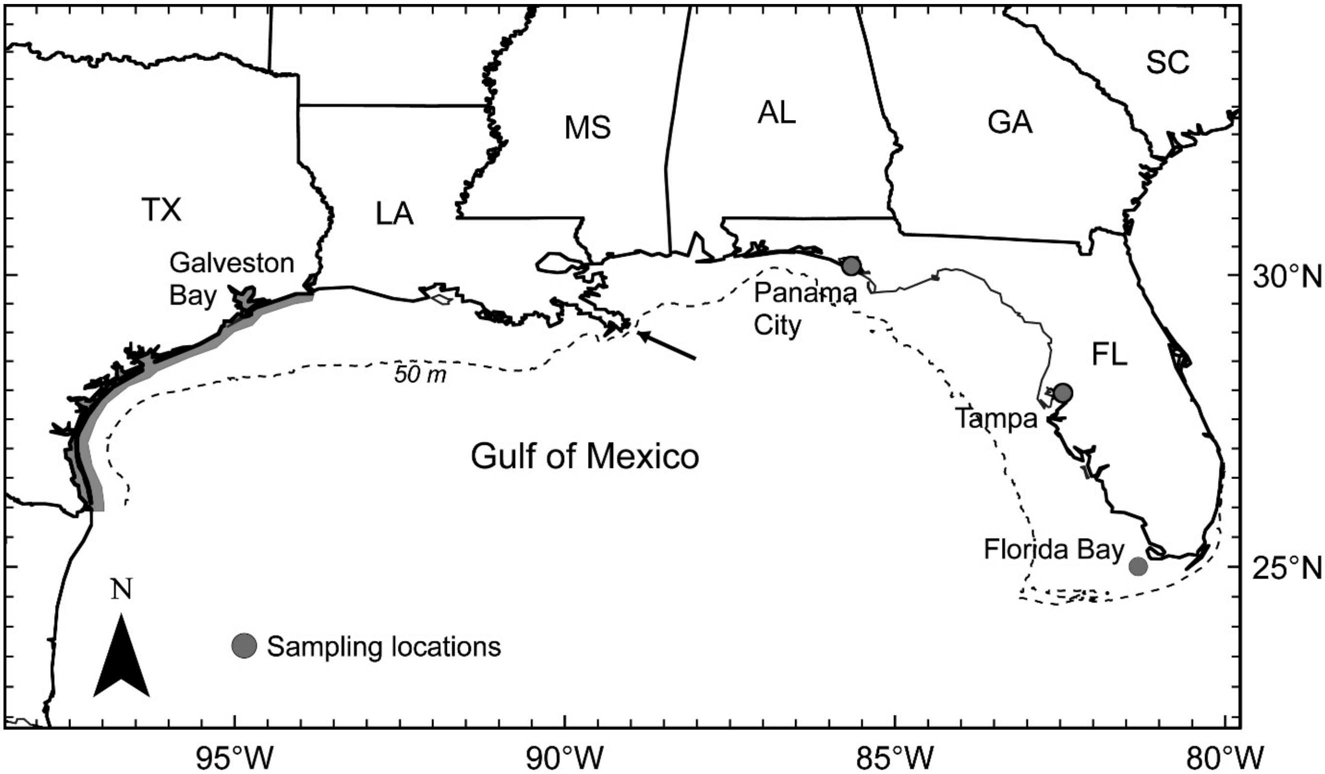

Fig. 1. Locations in the Gulf of Mexico where *Farfantepenaeus* sp. were collected. Arrow indicates the Mississippi River Delta, which separates the eastern and western Gulf.

identify the 'best' subsets of characters to discriminate taxa and predict species membership. GDA accounts for variability in each character and considers multiple characters simultaneously. GDA creates a model that attempts to minimize the likelihood of misclassification of new observations, and uses the Type VI sums of squares regression approach (StatSoft, 2004) identical to the effective hypothesis method of Hocking (2003). The effective hypothesis method provides an unambiguous and unique estimate of the variability of the outcome uniquely attributed to each variable, regardless of the number of variables analyzed, and minimizes the affect of redundancy in morphometric measurements. We evaluated the strength of the resultant 'best' character subsets with the multivariate Wilks' lambda test statistic, which measures the proportion of variance in each combination of characters unaccounted for by the model (StatSoft, 2004). We selected the 'best' suite of characters for the GDA models based on two criteria: discrimination power, i.e., > 85% of the variability between species accounted for by a given suite of characters, and, the ease of obtaining the suite of measurements included in the character subset.

We used a calibration data set composed of measurements made on shrimp from the western Gulf to estimate the classification functions for the 'best' subset of predictor variables, and then computed the misclassification or apparent error rate for an independent, cross-validation data set, i.e., cases not included in computation of parameter estimates for the calibration data set. Assessment of classification error yields an unbiased estimate of error, validates the model's ability to predict correctly group membership, and evaluates the performance of the classification functions (Moder et al., 2007). Models built by independent assessment of separate calibration and cross-validation data sets have better predictive ability for classification of new cases, and guards against over-fitting (StatSoft, 2004). We assumed 'equal' prior probabilities for predicting the identity of new cases because expected class sizes in natural populations are unknown and a correct answer has no associated cost (StatSoft, 2004).

GDA computes a set of classification functions for each species. Insertion of the resultant set of classification functions into the formula: Species = $a_{ij} + W_{1ij} * X_{1ij} + W_{2ij} * X_{2ij} + \ldots + W_{4ij} * X_{4ij}$ computes a separate classification score for each species. Once computed, the highest classification score, regardless of sign, predicts the identity of each new case. In the classification formula, 'a' represents the intercept value for either *F. aztecus* or *F. duorarum*; 'W' represents the classification function for the body part assessed; and 'X' represents the value of the raw, untransformed measurement in millimeters for that body part. The number subscript, i.e., 1, 2, 3, etc, represents the body part assessed, and the letter subscript 'i' or 'j' represents either *F. aztecus* or *F. duorarum.*

We used a suite of morphological characters compiled from the literature (Table 1) to identify a subset of *Farfantepenaeus* sp. (n = 153; mean: 4.9 mm CL; range: 1.9-7.0 mm CL; Table 2), whose identity had been verified molecularly to better evaluate the reliability of characters

Table 2. Number of *Farfantepenaeus duorarum* and *F. aztecus* < 7.0 mm CL from the Gulf of Mexico molecularly identified using a multiplex PCR assay and examined for differences in morphology. The Mississippi River Delta divides the eastern and western Gulf of Mexico. [1] Specimens used to calibrate and cross-validate the classification models. [2] Specimens used to test ability of classification models to discriminate *Farfantepenaeus* sp from the eastern Gulf. [3] Specimens used to assess reliability of characters that have been used to discriminate taxa.

Category and region	*F. duorarum*	*F. aztecus*	Totals
Molecularly identified			
Western Gulf of Mexico	49	91	140
Eastern Gulf of Mexico	29	1	30
Totals	78	92	170
Used in General Discriminant Analyses (GDA)			
Western Gulf of Mexico [1]	49	85	134
Eastern Gulf of Mexico [2]	29	1	30
Totals	78	86	164
Identified with morphological characters [3]			
Western Gulf of Mexico	32	91	123
Eastern Gulf of Mexico	29	1	30
Totals	61	92	153

Fig. 2. Generalized depiction of spinules along the epigastric and first dorsal tooth in a 3.2 mm CL *Farfantepenaeus duorarum.*

that have been used for species discrimination. We conducted the 'test' blind with collection location and season unknown to the identifier 'a priori' and compared the outcomes, i.e., species name assigned to the species identity verified by the multiplex PCR assay. We also compared the number of shrimp identified correctly by our GDA models with their molecularly verified identity to evaluate the performance of the classification functions and to assess model classification error. This approach permitted comparison of morphological, molecular, and GDA methodologies. Finally, in order to assess the performance and applicability of the classification models to other areas of the Gulf, we used the models calibrated with shrimp from the western Gulf to classify shrimp from the eastern Gulf.

We follow Pérez-Farfante (1969) and use CL as our length metric. Total length has traditionally been the length metric for penaeids, but TL can be difficult to measure due to specimen flexion and is impossible to measure in incomplete specimens. Consequently, comparison of our findings with the historical literature on young *F. duorarum* and *F. aztecus* required estimation of the TL:CL relationship for each species. We established the TL:CL relationship over the 1.9–7.0 mm CL size range examined in this study by linear regression as: $TL = -0.99 + 5.09*CL$ ($n = 73$, $R^2 = 0.99$) for *F. duorarum*, and $TL = -0.03 + 4.93*CL$ ($n = 90$, $R^2 = 0.99$) for *F. aztecus*.

Results

Overall, we molecularly identified 78 *F. duorarum* and 92 *F. aztecus* with about 80% of the 170 specimens collected in the western Gulf (Table 2). We collected *F. aztecus* during all months, except January and July (Appendix Table 2). Specimens of *Farfantepenaeus* sp. molecularly identified from the western Gulf in December, and late February to May contained only *F. aztecus*, whereas collections from August through November contained *F. aztecus* and *F. duorarum*. All *Farfantepenaeus* sp. molecularly identified from April, June and September collections in the eastern Gulf were *F. duorarum*, except for a 6.5 mm CL *F. aztecus* collected near Tampa Bay, Florida in late September (Appendix Table 2).

Collections in the western Gulf from May through November generally included a mixture of at least two and often all three species of commercially important and locally abundant penaeid shrimp that occur in U.S. waters of the Gulf of Mexico. Inclusion of PL and early juvenile *L. setiferus* in collections required recognition and elimination of their early life stages from the two-targeted species of *Farfantepenaeus* sp. Spinules along the dorsal carina of the sixth pleomere in both species of *Farfantepenaeus* and the absence of spinules in *L. setiferus* separate genera (Ringo and Zamora, 1968; Zamora and Trent, 1968). Young *Farfantepenaeus* sp. also had spinules on the epigastric and first DT, a character not previously reported for these two species (Fig. 2). *Farfantepenaeus duorarum* with five DT had 1-4 spinules on the epigastric tooth and 1-2 spinules on the first DT. *Farfantepenaeus aztecus* did not have spinules on the epigastric or first DT until they had 7 + 0 – 1 rostral teeth. Spinules increased in size and number with CL, and became easier to locate in *F. duorarum* and *F. aztecus* with a full complement of 8 + 2 rostral teeth. Both species of *Farfantepenaeus* ($n = 8$) usually had 5-10 spinules on the epigastric tooth and 3-8 spinules on the first DT by 4.0 mm CL.

Variability in morphology (expressed as the coefficient of variation, CV) generally decreased in *F. duorarum* and *F. aztecus* as development progressed. Overall, rostral characteristics exhibited the greatest variability, whereas length of the sixth pleomere and antennules peduncle segments exhibited the least variability, i.e., 5-10%. Total length of the second pereiopod varied by about 10-15% in each species, as did TL of the third pereiopod. Variability in spacing of DT generally decreased from 15-25% in shrimp $<$ 3.5 mm CL to 10–12% in shrimp $\geq$ 3.5 mm CL. Variability in placement of the VT, the distance between the rostrum tip and adjacent DT, and the distance between the rostrum tip and adjacent VT generally exceeded 20% in both species of *Farfantepenaeus*, regardless of CL.

Rates of development differed between seasons, as well as within and between species. Early PL's of *F. aztecus* with four DT collected in the western Gulf during September were smaller than PL's of *F. aztecus* with four DT collected in December and February (Table 3). When *F. duorarum* and *F. aztecus* with five DT were collected at the same location in August, *F. duorarum* were smaller than *F. aztecus*. Likewise, *F. duorarum* generally had more rostral teeth than did *F. aztecus* of comparable size (Table 3). *Farfantepenaeus duorarum* had a rostral tooth count of 7 + 1 as early as 2.3 mm CL (Table 3, Fig. 3), and consistently had at least 7 + 1 rostral teeth by about 2.5 mm CL. The smallest *F. duorarum* with 8 + 2 rostral teeth was 2.5 mm CL and all examined had a full complement of rostral teeth by 2.8 mm. Only two of 10 *F. aztecus* examined had one or two VT at $<$ 2.7 mm CL, and none had 8+2 rostral teeth at $<$ 3.0 mm CL (Table 3). No *Farfantepenaeus* sp. examined had nine DT until $\geq$ 3.5 mm CL (Table 3). Thirty-four of the 108 *Farfantepenaeus* sp. $\geq$ 3.5 mm CL had nine DT, and two *F. duorarum* and three *F. aztecus* had three VT.

Examination of the three antennal scale measurements suggest differences in relative shape of the scale between the two species of *Farfantepenaeus* ($n = 33$). *Farfantepenaeus duorarum* generally had an antennal scale about 10% longer than wide as measured from the base of the antennal spine, whereas scale height nearly equaled scale width in *F. aztecus* (Fig. 4). The length of the lateral spine adjacent to the antennal scale did not differ significantly between taxa, but the relatively greater height of the antennal scale in *F. duorarum*, i.e., about 25-30% longer than in *F. aztecus*, made the lateral spine look shorter in *F. duorarum* (Fig. 4).

Table 3. Rostral tooth development in *Farfantepenaeus aztecus* and *F. duorarum* from the western Gulf of Mexico. Length measurements in millimeters (mm). Specimens with four dorsal teeth included here for comparison purposes only. [1] Single specimen exhibiting rapid development. [2] Most specimens; hereafter have full complement of 8 + 2 rostral teeth. [3] Minimum size examined with nine DT. [4] Minimum size examined with three VT. [5] Smaller specimens from August.

Specimens examined	Months collected	Dorsal teeth	Ventral teeth	Mean TL (range)	Mean CL (range)
F. duorarum					
1	Sep.	4	0	8.9	1.8
6	Aug.-Sep.	5	0	9.1 (8.5-9.9)	1.9 (1.8-2.1)
1	Sep.	6	0	10.5	2.3
5	Aug.-Sep.	7	0-1	11.2 (10.6-11.5)	2.4 (2.3-2.5)
3	Sep.-Oct.	7	2	12.0 (11.6-12.3)	2.5 (2.5-2.6)
1	Sep.	8	1	12.0	2.5
2	Aug.-Sep.	8	2	11.5 [1]-13.5 [2]	2.5 [1]-2.8 [2]
1	Sep.	9	2	18.0 [3]	3.7 [3]
1	Nov.	8	3	24.2 [4]	4.9 [4]
F. aztecus					
2	Sep.	4	0	8.6 (8.2-9.0)	1.8 (1.8-1.9)
2	Feb.; Dec.	4	0	12.5 (11.5-13.6)	2.5 (2.3-2.6)
1	Aug.	5	0	11.3	2.3
2	Mar.	5	0	12.5 (12.4-12.5)	2.5 (2.4-2.6)
4	Nov.-Dec.	6	0	12.3 (12.1-12.4)	2.4 (2.3-2.5)
9	Oct.-Dec.	7	0-1	13.7 (12.6-15.7)	2.7 (2.5-3.2)
1	Oct.	7	2	12.9 [1]	2.6 [1]
4	Aug. [5]-Nov.	8	1	15.0 (14.4 [5]-15.7)	3.1 (2.9 [5]-3.3)
2	Sep.-Oct.	8	2	14.7 (14.6-14.7)	3.0; 3.2 [2]
2	Sep.-Oct.	9	2	15.5 [1]-16.7	3.5
1	Sep.	9	3	18.3 [4]	3.8 [4]

Recall that we measured scale height diagonally (Appendix Table 1), which made the height dimension longer than if measured vertically. If compared parallel to one another, the lateral spine approached or exceeded the tip of the antennal scale in *F. aztecus*, but reached only about 50-65% of scale height in *F. duorarum* (Fig. 4). Differences in antennal scale characteristics helped to discriminate *F. duorarum* and *F. aztecus* with < 7 – 8 + 2 rostral teeth, but not thereafter. The shape of the antennal scale sometimes differed slightly between the left and right sides of the body

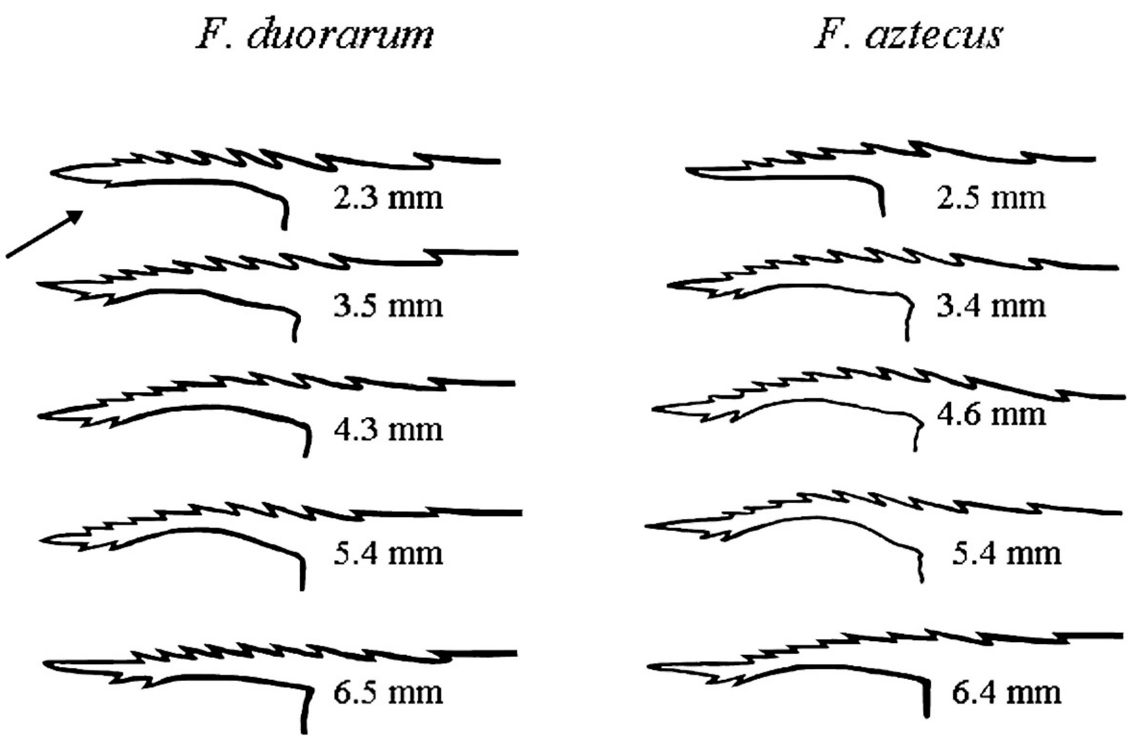

Fig. 3. Characteristic rostral shape patterns and placement of dorsal (DT) and ventral teeth (VT) in *Farfantepenaeus duorarum* and *F. aztecus*. Only teeth with the spinous tip 'free' from the shaft of the rostrum, i.e., not nubs, are counted. Total counts do not include the epigastric tooth. Arrow indicates position of first VT in 2.3 mm *F. duorarum*. Body size in mm CL. Note trident-shaped rostral tip in 5.4 mm *F. duorarum* and 4.6 mm *F. aztecus* with nine DT.

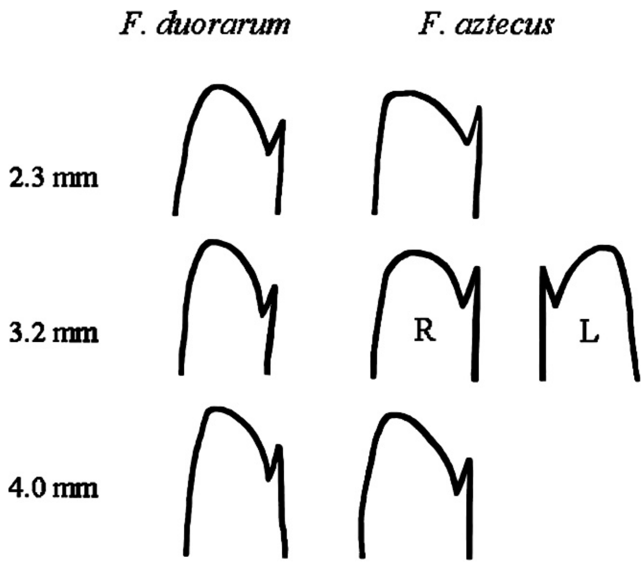

Fig. 4. Characteristic differences in shape of the antennal scale in *Farfantepenaeus duorarum* and *F. aztecus*. Body size in mm CL. Note difference in antennal scale shape between right and left sides of body in the 3.2 mm *F. aztecus*.

(Fig. 4; see 3.2 mm CL *F. aztecus* illustration) and both scales should be examined if one side appears ambiguous or intermediate in shape (Fig. 4).

Differences in morphology helped to discriminate *F. duorarum* and *F. aztecus*. At sizes < 2.5 mm CL, *F. aztecus* had a relatively wider gap between DT 4-5 and a relatively longer sixth pleomere than did *F. duorarum* (Tables 4-5). *Farfantepenaeus aztecus* had a sixth pleomere length measurement > 2.5 mm, whereas *F. duorarum* had a sixth pleomere length measurement < 2.5 mm (Table 5). Over the size range of 2.5-3.49 mm CL, *F. aztecus* had a relatively wider gap between DT 4-5; a relatively longer antennular peduncle, i.e., TL of first three segments of antennular peduncle; and, a relatively longer sixth pleomere than did *F. duorarum* of comparable size. A difference in antennal scale shape and in sixth pleomere length also separated *F. duorarum* and *F. aztecus* in the 2.5-3.49 mm CL size range with < 7 – 8 + 2 rostral teeth (Tables 4-5).

Reliable discrimination of *F. duorarum* and *F. aztecus* > 3.5 mm CL required four body measurements. At sizes of 3.5-4.49 mm CL, *F. duorarum* had a relatively wider gap between the epigastric tooth and DT 4 and between DT 2-3; a relatively longer second antennular peduncle segment; and, a relatively longer third pereiopod than did *F. aztecus* (Table 4). At sizes of 4.5-5.49 mm CL, *F. duorarum* had a relatively wider gap between DT 2-5, whereas *F. aztecus* had a relatively wider gap between the epigastric tooth and posterior margin of the carapace and between VT; and, a relatively greater distance between the rostrum tip and proximal VT (Table 4). At sizes of 5.5-7.0 mm CL, *F. aztecus* had a relatively greater distance between DT 1 and the posterior margin of the carapace, the epigastric tooth and DT 4, the rostrum tip and adjacent VT, and had a relatively longer second pereiopod than did *F. duorarum* (Table 4).

Comparison of Methods

We examined the morphology of 153 *Farfantepenaeus* sp. from across the Gulf (Table 2) whose species identity had been verified using a multiplex PCR assay to evaluate the reliability of characters that have been used for species discrimination (Table 1). Overall, use of 'traditional' characters resulted in misidentification of 31% of the shrimp, with 26 molecularly verified *F. aztecus* identified morphologically as *F. duorarum*, and 22 molecularly verified *F. duorarum* identified morphologically as *F. aztecus*. Relative to the total number of specimens examined for each species, use of 'traditional' characters resulted in misclassification of about 35% of *F. duorarum* and 28% of *F. aztecus*. Regionally, 12 of 30 *F. duorarum* from the eastern Gulf, and 10 of 32 *F. duorarum* from the western Gulf were misidentified as *F. aztecus* based on morphology. Similarly, 25 of 91 *F. aztecus* from the western Gulf were misidentified morphologically as *F. duorarum*. One *F. aztecus* from the eastern Gulf was identified as *F. duorarum*.

Our GDA models accurately identified ≥ 90% of the *Farfantepenaeus* sp. from the western Gulf in each of the five data sets, and 94.8% of the 134 specimens in the combined calibration and cross-validation data sets (Table 4). We examined an all-inclusive calibration data set as well as alternate data sets composed of *Farfantepenaeus* sp. from the western Gulf that differed only in the seasonal distribution of individuals and achieved the 'best' species discrimination with shrimp collected from February through May and September through mid-December in the final calibration data sets. Of the 134 shrimp from the western Gulf used to build the classification models, the final subsets of functions misidentified a total of seven, with four of the specimens collected in June and August.

When we applied the classification models built with *Farfantepenaeus* sp. from the western Gulf to the 30 *Farfantepenaeus* sp. ≥ 2.5 mm CL from the eastern Gulf the models correctly classified only 11 of the 30 or 36.7% overall. We also measured the sixth pleomere length of four shrimp < 2.5 mm CL collected in Florida Bay, an area dominated by *F. duorarum*. Although their identity was not molecularly verified due to unknown preservation history, all four had a sixth pleomere measurement < 2.5 mm, which identified them as *F. duorarum*. The model classified correctly all three *Farfantepenaeus* sp. in the 2.5-3.49 mm CL size interval as *F. duorarum*. Application of the models to the three size categories ≥ 3.5 mm CL (n = 27) classified correctly only 29.6% of these larger shrimp as *F. duorarum*.

Discussion

We verified the species identity of PL and early juvenile *F. aztecus* and *F. duorarum* 1.8–7.0 mm CL using a multiplex PCR assay as described in Alvarado Bremer et al. (2010) and examined these specimens for differences in morphology. Our combined morphological and molecular approach identified new characters to discriminate *F. duorarum* and *F. aztecus* with five or more DT. Spinules on the epigastric and first DT in *F. duorarum* and *F. aztecus* and the absence

Table 4. Discriminant coefficients for the 'best' suite of characters to discriminate *F. duorarum* and *F. aztecus* < 7.0 mm CL from the western Gulf of Mexico. Wilks' lambda is the amount of variability unexplained by the model. Higher standardized coefficient values indicate a greater contribution to species discrimination, regardless of sign. For new observations, insert the classification functions into the discriminant equation to compute a classification score for each species. The highest classification score, regardless of sign, predicts the identity of each new case. Species = $a_{ij} + W_{1ij} * X_{1ij} + W_{2ij} * X_{2ij} + \ldots + W_{4ij} * X_{4ij}$ where 'a' represents the intercept value for either *F. aztecus* or *F. duorarum*; 'W' represents the classification function for the body part assessed; and, 'X' represents the value of the raw, untransformed measurement in millimeters for the same body part. The number subscript, i.e., 1, 2, 3, etc, represents the body part assessed; and, the letter subscript 'i' or 'j' represents either *F. aztecus* or *F. duorarum.* Abbreviations for body measurements: carapace length (CL), total length (TL), epigastric tooth (ET), dorsal tooth (DT), ventral tooth (VT), carapace termination (CT); rostral tip to adjacent VT (RT-aVT); rostral tip to proximal VT (RT-pVT); distance between VT 1-2, or VT 2-3 if three VT present (VT 1-2); length of second antennular segment (AS 2 L); TL of 1st three antennular segments (AS 1-3 TL); TL of second pereopod (Leg 2 TL); TL of third pereiopod (Leg 3 TL); and sixth pleomere length (Abd 6 L).

Size interval (mm CL)	*F. duorarum*	*F. aztecus*	Model Wilks' lambda	Number used: *F. duorarum*/*F. aztecus*			Coefficients	Species
	mm TL (approx.)			Calibration sample % correct	Validation sample % correct	Overall predicted % correct		
< 2.5	< 11.5	< 12.5	10%	6/5	5/2	11/7	Standardized	
				100%	100%	100%	Classification functions	*F. duorarum*
								F. aztecus
2.5-3.49	11.5-16.8	12.5-17.0	15%	8/7	6/16	14/23	Standardized	
				100%	96%	97%	Classification functions	*F. duorarum*
								F. aztecus
3.5-4.49	17.0-21.4	15.5-22.0	8%	7/7	0/11	7/18	Standardized	
				100%	91%	96%	Classification functions	*F. duorarum*
								F. aztecus
4.5-5.49	18.1-27.4	21.6-27.4	12%	7/8	3/7	10/15	Standardized	
				100%	80%	92%	Classification functions	*F. duorarum*
								F. aztecus
5.5-7.0	27.0-34.4	26.8-36.7	15%	7/7	0/15	7/22	Standardized	
				100%	80%	90%	Classification functions	*F. duorarum*
								F. aztecus

thereof in *L. setiferus* provide a new character by which to separate genera in areas where species distributions overlap. We found some characters traditionally used for species discrimination to be unreliable.

Relative differences in length of the sixth pleomere and in shape of the antennal scale reliably discriminate early PL's of *F. duorarum* and *F. aztecus* with < 7 – 8 + 2 rostral teeth. *Farfantepenaeus duorarum* have an acutely rounded antennal scale that is longer compared to the height of the adjacent lateral spine than do *F. aztecus* of comparable size (Fig. 4) as originally described by Williams (1959). Although we included only shrimp with five or more DT in analyses, differences in antennal scale shape reliably discriminate *F. duorarum* and *F. aztecus* with four DT that we examined. Chuensri (1968) used a sixth pleomere length measurement of 2.65 mm as the cut-off point to discriminate *F. aztecus* from *F. duorarum* compared to our cut-off of 2.5 mm. Chuensri (1968), however, did not verify the species identity of his wild-caught shrimp and collections from south Florida may include other local species. Nevertheless, the differences in antennal scale shape are consistent among locations and seasons and improves the reliability of species discrimination in early PL's with < 7 – 8 + 2 rostral teeth, especially for those individuals where the sixth pleomere measurement falls near the 2.5 mm cut-off point.

Farfantepenaeus duorarum have more rostral teeth than *F. aztecus* of comparable size (Table 3). Early PL's of wild-caught *F. duorarum* from the western Gulf and those lab-reared (Dobkin, 1961) consistently have one VT by about 2.3 mm CL, and 7 – 8 + 1 – 2 rostral teeth by about 2.5 mm CL. Wild-caught *F. duorarum* from south Florida have a full complement of rostral teeth (8 + 2) at an average size of 2.3 mm CL (Chuensri, 1968). Wild-caught *F. aztecus* do not have the first VT until ≥ 2.5 mm CL, and typically, do not have a full complement of rostral teeth until ≥ 2.9 mm CL (Table 3).

Our data do not support the qualitative observation of Williams (1953) that differences in relative shape of the rostrum helps to discriminate *F. duorarum* and *F. aztecus* as small as 3.5 mm CL. Williams (1953) urged caution when applying this character to shrimp < 10 mm CL, but suggested that when ''viewed laterally, the entire rostrum is usually shorter in relation to its depth'' in *F. duorarum* than in *F. aztecus*. While *F. aztecus* 4.5-7.0 mm CL generally has a longer rostrum than do *F. duorarum* of comparable length, intra-specific variability in rostrum length (RL; overall mean CV: 10% in each species) makes this character unreliable for species discrimination. Likewise, rostrum depth at the fifth DT varies by an overall mean CV of 15% in each species, which makes the RL to rostrum depth relationship problematic for species discrimination at sizes < 7.0 mm CL. Williams (1953) suggestion that *F. aztecus* generally has a more attenuated rostrum tip dorsally than do *F. duorarum* of comparable size also differs from what we observed. Attenuation of the rostrum tip depends on the total number of DT. Recall that 31.5% of the wild-caught shrimp ≥ 3.5 mm CL examined had nine DT. A higher number of DT decreases the distance between the anterior DT and rostrum tip. In specimens with nine DT, the tip of the rostrum appears more like a 'trident' with the anterior DT and anterior VT in opposition (Fig. 3). We estimate that another 5% or more of wild-caught shrimp have a malformed rostrum. If the sample of shrimp ≥ 3.5 mm CL that we examined is representative of the wild population, at least one-third of *F. duorarum* and *F.*

Table 4. Extended.

Intercept	RT – aVT	RT – pVT	VT 1–2	DT 2–3	DT 4–5	DT 2–5	ET - DT 4	ET - CT	DT 1 - CT	AS 2 L	AS 1–3 TL	Leg 2 TL	Leg 3 TL	Abd 6 L
0.0					−0.6									1.1
−78					20									71.7
−146					−112									106
0.0					1.6						−1.9			−0.9
−308					−636						209			161
−424					−757						245			190
0.0				−6.5			7.3			−5.0			4.2	
−273				−2459			555			−1052			162	
−152				−1691			389			−746			116	
0.0		−1.2	1.3			−2.4		2.5						
−151		−85	76			122		57						
−156		−131	262			−0.4		115						
0.0	−2.3						5.3		−5.4			1.8		
−143	38						−106		148			−18		
−178	82						−195		219			−26		

aztecus have nine DT, and cannot be reliably discriminated by the reported difference in attenuation of the rostrum tip.

Variability in timing of character change due to seasonal differences in rates of growth and development make species discrimination more difficult in areas where distributions overlap temporally and spatially. *Farfantepenaeus aztecus* develop faster and characters change more rapidly during late summer than during spring, a pattern consistent with that for *F. duorarum* (Williams, 1959; Criales et al., 2003). Seasonal differences in rates of development is not surprising given the inverse relationship between development time and water temperature is well known (Hartnoll, 1982; Williamson, 1982; Dall et al., 1990b). Higher water temperatures generally increase metabolism and the frequency of molting, which shortens the intermolt interval and increases the rate of growth and development (Teinsongrusmee, 1965; Dall et al., 1990b; Smith, 1997). The fact that environmental conditions can induce differences in the rate of development suggests that the maximum size, i.e., CL, at which characters reliably discriminate taxa change with water temperature. Therefore, the number of rostral teeth may be a better basis for inter-species comparisons in early PL's than body length. For example, a rostral tooth count of 7 – 8 + 1 is the upper limit at which differences in sixth pleomere length and antennal scale shape reliably discriminate taxa. Thereafter, the sixth pleomere becomes relatively shorter in *F. aztecus* and measurements overlap with *F. duorarum*. Antennal scale shape characteristics also change in *F. aztecus* with 7 – 8 + 2 rostral teeth (Fig. 4). Species discrimination thereafter requires body measurements.

Table 5. Discrimination of *Farfantepenaeus duorarum* and *F. aztecus* postlarvae with five or more and < 7 – 8 + 2 rostral teeth based on a measurement cut-off value of 2.5 mm for sixth abdominal segment length. All measurements are raw and untransformed. The Mississippi River Delta divides the eastern and western Gulf of Mexico.

Measurement	*F. duorarum* (n = 9)	*F. duorarum* (n = 19)	*F. aztecus* (n = 21)
Location	Eastern Gulf	Western Gulf	Western Gulf
Total length (mm)			
Mean	11.0	10.8	13.0
Range	9.1-14.1	8.5-12.5	11.3-15.7
Carapace length (mm)			
Mean	2.4	2.3	2.6
Range	2.1-2.8	1.8-2.7	2.2-3.2
Sixth pleomere length (mm)			
Mean	2.2	2.1	2.8
Range	2.1-2.3	1.9-2.3	2.6-3.2
95% confidence interval	2.0-2.4	2.0-2.2	2.7-2.9

We selected 'best' subsets of characters relatively easy to measure that reduce the time necessary to manipulate and identify the large number of specimens routine monitoring surveys require because the most useful characters require minimal specimen handling (Rothlisberg et al., 1983). We limited final subset size to four or fewer metrics because more characters extend the time required to examine each specimen, but minimally improve a model's ability to classify cases and predict species membership (Moder et al., 2007). We grouped specimens into size intervals to minimize developmental differences and reduce morphological variation that can hinder species discrimination. Recall that our discriminant models built on smaller intervals of size explained ≥ 85% of the variability between taxa. When we included all *F. duorarum* and *F.*

Table 6. Body measurements (mean ± 95% confidence intervals) important in species discrimination of *Farfantepenaeus duorarum* and *F. aztecus* > 2.5 mm CL from the western Gulf of Mexico. Use measurements as a guide to identify outliers before inserting the classification functions into the discriminant equation. Abbreviations: ventral teeth (VT); dorsal teeth (DT); epigastric tooth (ET). [1] Measured between VT 2-3 when three VT present. [2] Measurement only useful for postlarvae with < 7 – 8 + 2 rostral teeth.

Measurement	2.5-3.49 mm CL	3.5-4.49 mm CL	4.5-5.49 mm CL	5.5-7.0 mm CL
Count (*F. duorarum*/*F. aztecus*)	14/23	7/16	8/13	7/14
Total length (TL)	14.0 (11.5-17.0)	19.0 (16.3-22.0)	24.5 (18.1-27.5)	31.2 (26.7-36.2)
Carapace length (CL)	2.9 (2.5-3.5)	3.9 (3.5-4.4)	5.0 (4.5-5.4)	6.3 (5.6-7.0)
Rostrum tip to adjacent VT				1.0 (0.9-1.2)
Rostrum tip to proximal VT			1.1 (1.0-1.2)	
Distance between VT [1]			0.4 (0.3-0.4)	
Distance between DT 2-3		0.4 (0.3-0.5)		
Distance between DT 4-5	0.2 (0.2-0.3)			
DT 1 to carapace termination				5.2 (4.9-5.4)
Distance between DT 2-5			1.5 (1.4-1.6)	
ET to end of carapace			3.1 (3.0-3.2)	
ET to DT 4		1.3 (1.2-1.4)		3.4 (3.3-3.6)
Length of second segment of antennular peduncle		0.6 (0.5-0.7)		
TL of 1st three segments of antennular peduncle	2.1 (2.0-2.2)			
TL of second pereiopod				7.3 (6.9-7.7)
TL of third pereiopod		5.6 (5.2-5.9)		
Sixth pleomere length [2]	2.5 (2.2-2.8)			

aztecus 3.5-7.0 mm CL into a single model, four metrics explained only 53% inter-species variability.

We did not examine differences in shape of the external genitalia, i.e., thelycum and petasma, described by Pérez-Farfante (1970) because this type of observation requires tedious and time-consuming manipulation, and often excision of abdominal appendages. However, external genitalia were included in the suite of traits used to evaluate the reliability of 'traditional' characters for species discrimination (Table 1). Given a misclassification rate of 31% when external genitalia were used as one of the primary criteria to discriminate early juveniles of *Farfantepenaeus* sp. ideas about the distribution patterns and habitat preferences of *F. duorarum* and *F. aztecus* may require re-evaluation.

Classification models accurately discriminate > 90% of *F. aztecus* and *F. duorarum* ≥ 3.5 mm CL from the western Gulf and increase the reliability of species identification by at least 20% over 'traditional' characters (Table 1). Elimination of specimens of *Farfantepenaeus* sp. that fall outside of confidence intervals that encompass 95% of the mean for a character identified as important for species discrimination will further reduce misclassification error (Table 6). The final identity of such specimens should remain at the generic level, although species identity could be determined genetically.

The unsatisfactory performance of the classification models in discriminating *Farfantepenaeus* sp. ≥ 3.5 mm CL from the eastern Gulf may reflect the cumulative impact of different environmental conditions encountered during development, the onset of sexual dimorphism, and/or high genetic diversity among populations (McMillen-Jackson and Bert, 2004). *Farfantepenaeus duorarum* and *F. aztecus* may also hybridize, though to our knowledge, hybridization has not been reported in nature. Several other species of penaeids have hybridized in the laboratory (Bray et al., 1990; Benzie et al., 1995), but the likelihood of hybridization in the wild is small. Adults display low levels of behavioral interaction during inter-species crosses, and the fertility, hatch rate and survivorship of hybridized progeny to the PL stage is poor (Benzie et al., 1995; Misamore and Browdy, 1997).

Genetic diversity may help explain the relatively high morphological plasticity between populations of *Farfantepenaeus duorarum* in the eastern and western Gulf. Populations of *F. aztecus* and *F. duorarum* along the U.S. Atlantic and Gulf coasts show no significant phylogenetic structure or population subdivision based on mtDNA diversity (McMillen-Jackson and Bert, 2003; McMillen-Jackson and Bert, 2004). Genetic studies, however, often do not support population differences that imply prolonged ecological separation or rapid changes in phenotypic traits (Begg et al., 1999; Swain and Foote, 1999; Waples et al., 2008). Significant differences in population genetic structures imply evolutionary time, not the ecological time over which changes in the phenotype may occur (Palumbi, 2003; Waples et al., 2008; Reiss et al., 2009). Discrete populations of the same species that differ morphologically between the eastern and western Gulf is an increasingly common pattern normally associated with hydrologic conditions that restrict gene flow across the Mississippi River (Felder and Staton, 1994), as evidenced by disparate populations of *L. setiferus* (McMillen-Jackson and Bert, 2003). The haplotype and nucleotide diversities displayed by *F. duorarum* are among the highest for any decapod (McMillen-Jackson and Bert, 2004), which is consistent with the possibility of different ecological populations of *F. duorarum* in the eastern and western Gulf that may warrant further study.

In closing, understanding the life cycle, variability, ecological niche, and a species role in the community and ecosystem requires a multidisciplinary approach (Will and Rubinoff, 2004; Ebach and Holdrege, 2005; Boero, 2010). While molecular techniques permit verification of species identity regardless of life stage and improve our ability to discriminate taxa and assess differences, 'DNA specimens' still require 'traditional' approaches to taxonomy to make specimens morphologically distinguishable (Ebach and

Holdrege, 2005) for identification in the field and laboratory, and for inclusion in taxonomic keys and field guides. Integration of molecular taxonomy and comparative morphology, as we did here, can provide insight into patterns of diversity and the ecological and evolutionary principles that encompass fisheries management.

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Appendix Table 1. Body measurements taken on young *Farfantepenaeus aztecus* and *F. duorarum* from the Gulf of Mexico. Inter-tooth distances were measured between anterioventral margins of teeth near the base. Abbreviations: epigastric tooth (ET); dorsal tooth (DT); ventral tooth (VT). [1] Measured to eighth DT if > 8 DT present. [2] Measured to second VT if three present. [3] Measured between VT 2–3 if three VT present.

Measurement	Definition
Total length (TL)	Between rostrum tip and proximal margin of telson following body contour (not a straight linear distance)
Carapace length (CL)	Postorbital margin to termination of carapace along dorsal midline
Rostrum length (RL)	Tip of rostrum to postorbital margin of carapace
ET to termination of carapace	ET to termination of carapace along dorsal midline
ET to DT 1	ET to DT 1
ET to DT 2	ET to DT 2
ET to DT 4	ET to DT 4
DT 1 to termination of carapace	DT 1 to termination of carapace along dorsal midline
DT 3 to termination of carapace	DT 3 to termination of carapace along dorsal midline
DT 5 to termination of carapace	DT 5 to termination of carapace along dorsal midline
DT 1-2	DT 1 to DT 2
DT 2-3	DT 2 to DT 3
DT 3-4	DT 3 to DT 4
DT 4-5	DT 4 to DT 5
DT 1-3	DT 1 to DT 3
DT 1-5	DT 1 to DT 5
DT 2-4	DT 2 to DT 4
DT 2-5	DT 2 to DT 5
DT 3-5	DT 3 to DT 5
Rostrum tip to adjacent DT [1]	Tip of rostrum to adjacent DT
Rostrum tip to adjacent VT [2]	Tip of rostrum to adjacent VT
Rostrum tip to proximal VT	Tip of rostrum to posteriormost VT
Proximal VT to postorbital margin	Posteriormost VT to postorbital margin of carapace
Distance between VT [3]	Anterior to posterior VT
Rostrum depth at DT 5	Vertical depth of rostrum at anterior margin of DT 5
Length of antennal scale spine	Diagonal distance from tip of lateral spine to intersection of spine and scale as measured along inner edge of spine
Height of antennal scale	Diagonal distance from intersection of antennal scale and lateral spine to highest point of scale margin
Width of antennal scale	Horizontal distance from intersection of antennal scale and lateral spine to opposite margin of scale
Length of first segment of antennular peduncle	Proximal to distal margins of first antennular segment
Length of second segment of antennular peduncle	Proximal to distal margins of second antennular segment
Length of third segment of antennular peduncle	Proximal to distal margins of third antennular segment
Total length of antennular peduncle segments	Proximal margin of first segment to distal margin of third segment
Chela length of second pereopod	Proximal to distal margins of chela
TL of second pereopod	Distal margin of chela to proximal margin of ischium
Chela length of third pereopod	Proximal to distal margins of chela
TL of third pereopod	Distal margin of chela to proximal margin of ischium
Length of sixth abdominal segment	Anterior to posterior margins of sixth abdominal segment

Appendix Table 2. Distribution of *Farfantepenaeus duorarum* and *F. aztecus* from the Gulf of Mexico molecularly identified using a multiplex PCR assay and examined for differences in morphology. Specimens from the eastern Gulf were collected in April, June and September only. Neither species collected in January or July. The Mississippi River Delta divides the eastern and western Gulf of Mexico.

											Totals	
mm CL	Feb.	Mar.	Apr.	May	Jun.	Aug.	Sep.	Oct.	Nov.	Dec.	Western Gulf	Eastern Gulf
F. duorarum												
< 2.5						1	11		1		13	
2.5-3.49					3	2	10	2			14	3
3.5-4.49			1		3		4		3		7	4
4.5-5.49			8		3	1	3	1	6		8	14
> 5.5-7.0			5		1		2	4	3		7	8
F. aztecus												
< 2.5	1	1				1	2		4	2	11	
2.5-3.49		1	1			1	3	11	5	1	23	
3.5-4.49			2			1	6	7	2		18	
4.5-5.49			2	4		2		1	4	2	15	
> 5.5-7.0			3	5	3		1	2	4		17	1